Supplemental Materials

RhoA orchestrates glycolysis for Th2 cell differentiation and allergic airway inflammation

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Supplemental Methods

Cytokine Assay

Cytokines in the culture supernatants and BAL fluid were measured by ELISA. IL-4, IL-5 and IFN-γ were measured with OptEIA kits (BD Bioscience); IL-13, IL-17 and eotaxin were measured with DuoSet ELISA kits (R&D Systems, Minneapolis, MN); TGF-β1 was assayed by TGF-β1 E_{max} ImmunoAssay System (Promega, Madison, WI). ELISA plates were developed with TMB substrate (BD Bioscience), and read with a microplate reader (Molecular Devices, Sunnyvale, CA). Cytokine mRNA levels were measured by real-time quantitative PCR.

Real-time PCR

Total RNA was extracted from lung tissues or from cultured cells with the RNeasy Mini Kit (Qiagen, Valencia, CA), and cDNA was prepared by using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Quantitative PCR was performed with the Platinum SYBR Green qPCR SuperMix-UDG w/RO or TaqMan Gene Expression Master Mix (Life Technologies, Carlsbad, CA) on a Mastercycler ep realplex4 apparatus (Eppendorf, Westbury, NY). The data were normalized to the 18S reference. Primers for IL-4, IL-5, IL-13, eotaxin, MUC-5AC, and Gob-5 were designed with OLIG 4.0 software as reported.[1]

Western Blot

Cells were either untreated or stimulated with anti-CD3/CD28 for 10 min to 17 h. For whole-cell lysates, cells were extracted with RIPA lysis buffer (1× PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM phenyl methyl sulfonyl fluoride, and protease inhibitors). Lysates were resolved by SDS-PAGE, then electrophoretically transferred onto a nitrocellulose membrane (GE Healthcare, Piscataway, NJ) and incubated with the antibodies against Stat1 (E-

23), Stat6 (S-20) (Santa Cruz, Dallas, TX), phospho-Stat1 (Tyr701) (58D6), phospho-Stat6 (Tyr641), phospho-S6K (Thr389) , phospho-4E-BP (Thr37/46, 236B4) , phosphor-Akt (S473, D9E) , phospho-PKCθ (Thr538), phospho-LIMK1/2 (Thr508/505) , phospho-MLC2 (Ser19) RhoA (67B9) (Cell Signaling, Danvers, MA); or β-actin (AC-15) (Sigma). The bands were visualized with the enhanced chemiluminescence system (Thermo Scientific).

Generation of RhoGAP$^{-/-}$ chimeric mice

Because RhoGAP$^{-/-}$ mice are embryonically lethal, embryonic day 14.5 fetal liver cells proficient (RhoGAP$^{+/+}$) or deficient (RhoGAP$^{-/-}$) for RhoGAP were collected from RhoGAP$^{+/-}$ breeding parents. The cells were then transplanted into lethally-irradiated syngeneic BoyJ mice. Two month later, a cohort of the chimeric mice was sacrificed and CD4^{+} naïve T cells were isolated and analyzed for Th1 and Th2 cell differentiation. The rest of the chimeric mice were examined for allergic airway inflammation.

Statistical analysis

All experimental data were analyzed and compared for statistically significant differences using two-tailed Student's *t* or Mann-Whitney *U* test, and a *P* value of < 0.05 was considered significant.

Supplemental References:

1. Yang JQ, Liu H, Diaz-Meco MT, Moscat J. NBR1 is a new PB1 signalling adapter in Th2 differentiation and allergic airway inflammation in vivo. EMBO J 2010; 29:3421-33.

Supplemental Figure Legends

Fig E1. RhoA deficiency impairs T cell homeostasis. **A**, Immunoblot of RhoA expression in splenic T cells from WT and $RhoA^{-/-}$ mice. **B**, Flow cytometry analysis of splenic $CD4^+$ and $CD8^+$ T cells. Right panel shows proportions and absolute numbers of $CD4^+$ and $CD8^+$ T cells (n=13 mice per group). **C**, Flow cytometry analysis of $CD4^+$ and $CD8^+$ naïve and memory-phenotype T cells. Right panels show proportions and absolute numbers of naïve ($CD44^{lo}CD62L^{hi}$), effector memory (T_{EM}, $CD44^{hi}CD62L^{lo}$) and central memory (T_{CM}, $CD44^{hi}CD62L^{hi}$) T cells (n=5 mice per group). **D**, Absolute numbers of non-T cell populations in spleen (n=5 mice per group). Data are representative of two to three independent experiments. Error bars represent SD. **P* < .05, ***P* < .01.

Fig E2. RhoA deficiency dampens mTORC2 but not mTORC1 activation. WT and $RhoA^{-/-}$ (KO) $CD4^+$ naïve T cells were cultured with or without anti-CD3/CD28 for 1h. Phosphorylated (p) S6K, 4E-BP, Akt, and PKCθ were examined by immunoblot. β-actin was blotted as loading control.

Fig E3. RhoGAP deficiency/RhoA gain-of-function promotes Th2 cell differentiation and allergic airway inflammation. A, RhoGAP deficiency causes an enhanced RhoA signaling activation. $CD4^+$ T cells were examined for phopho (p) LIMK1/2 and MLC2 by immunoblot. β-actin was blotted as loading control. **B,** RhoGAP deficiency has no effect on Th1 cell differentiation. $CD4^+$ naïve T cells were cultured under Th1-skewed conditions for 4 days and restimulated with PMA plus ionomycin for 5 h. Supernatants were collected for ELISA assays to detect IFN- γ secretion. **C,** RhoGAP deficiency promotes Th2 cell differentiation. $CD4^+$ naïve T cells were cultured under Th2-skewed conditions for 4 days and restimulated with PMA plus

ionomycin for 5 h. Supernatants were collected for ELISA assays to detect IL-4 and IL-5 secretion. **D-G,** RhoGAP deficiency promotes OVA-induced allergic airway inflammation. WT and RhoGAP$^{-/-}$ mice were immunized i.p. with OVA and then challenged with aerosolized OVA or PBS as control. Mice were sacrificed 24 h after the last challenge. Total BAL cells and differential cell counts (**D**), representative Kwik-Diff staining for BAL cytospins (**E**) and H&E staining of lung tissue sections (**F**), and levels of cytokines in BAL fluids (**G**) are shown. For **B** and **C**, CD4^{+} naïve T cells were pooled from 3 mice. Error bars represent SD of triplicates. For **D** and **G**, Error bars represent SE of 3-5 mice. **P* < .05, ***P* < .01.

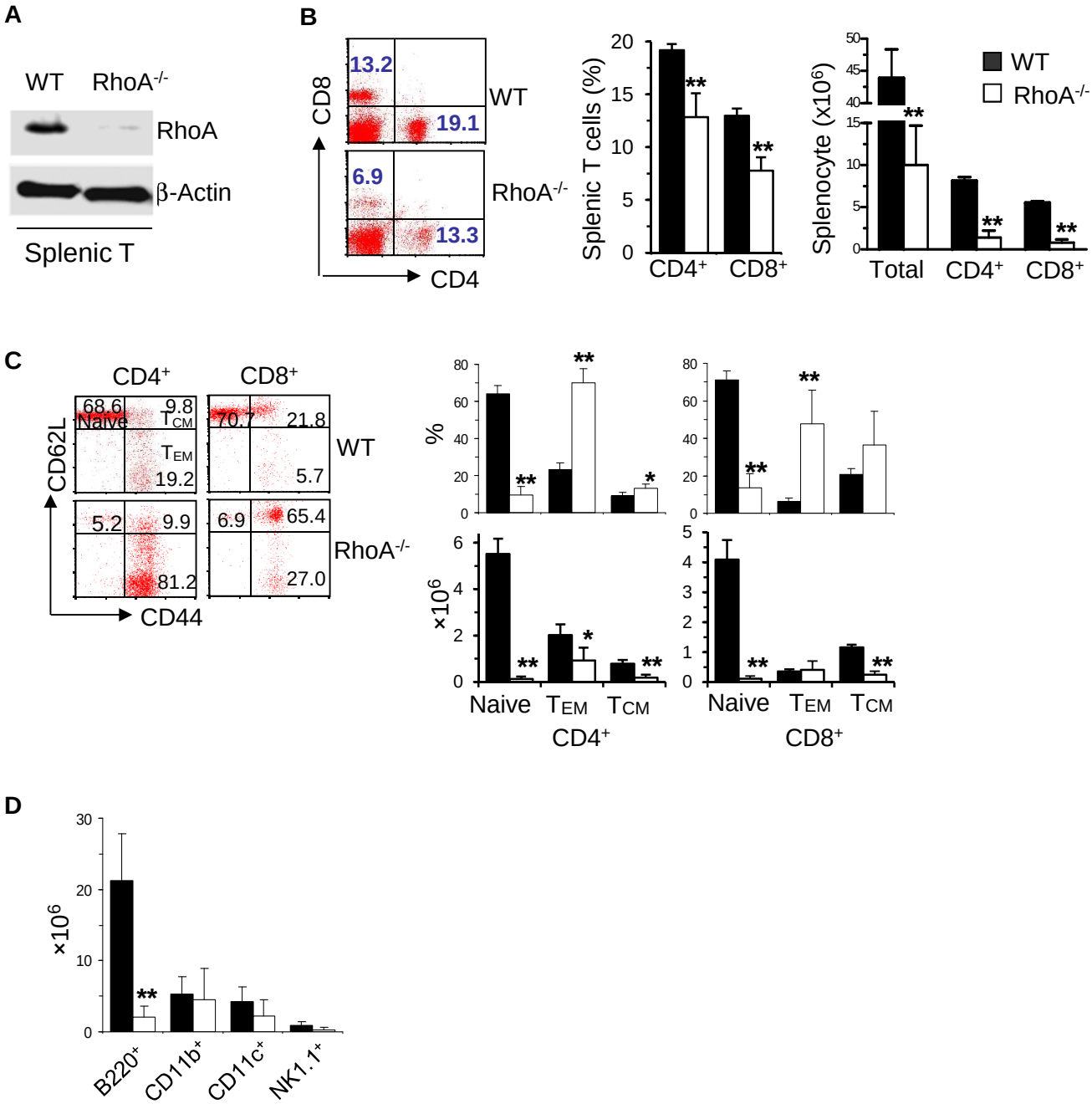
A
WT
RhoA-/-
RhoA
β-Actin
Splenic T
B
CD8
CD4
13.2
19.1
6.9
13.3
WT
RhoA-/-
Splenic T cells (%)
CD4+
CD8+
Splenocyte (x10^6)
Total
CD4+
CD8+
WT
RhoA-/-
C
CD4+
CD8+
CD62L
CD44
68.6
Naive
9.8
TCM
TEM
19.2
70.7
21.8
5.7
5.2
9.9
81.2
6.9
65.4
27.0
WT
RhoA-/-
%
×10^6
Naive
TEM
TCM
CD4+
CD8+
D
×10^6
B220+
CD11b+
CD11c+
NK1.1+

Fig. E1

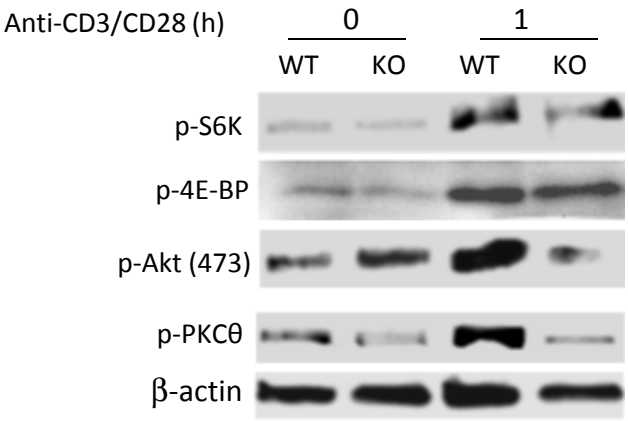

Fig. E2

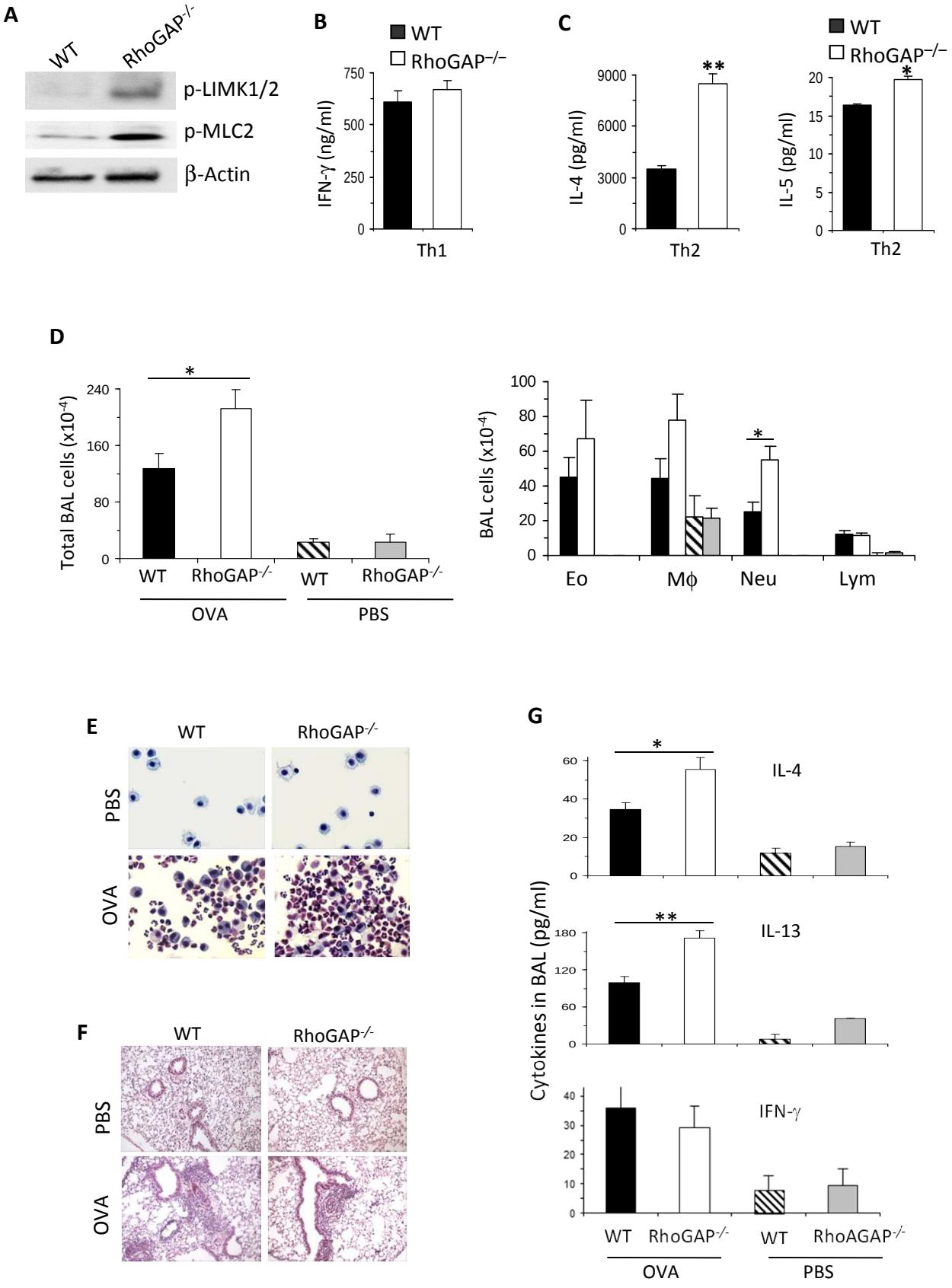
A
WT
RhoGAP-/-
p-LIMK1/2
p-MLC2
β-Actin
B
WT
RhoGAP−/−
IFN-γ (ng/ml)
750
500
250
0
Th1
C
WT
RhoGAP−/−
IL-4 (pg/ml)
9000
6000
3000
0
**
Th2
IL-5 (pg/ml)
20
15
10
5
0
*
Th2
D
Total BAL cells (x10-4)
240
160
80
0
*
WT
RhoGAP-/-
WT
RhoGAP-/-
OVA
PBS
BAL cells (x10-4)
100
80
60
40
20
0
*
Eo
Mϕ
Neu
Lym
E
WT
RhoGAP-/-
PBS
OVA
F
WT
RhoGAP-/-
PBS
OVA
G
Cytokines in BAL (pg/ml)
IL-4
60
40
20
0
*
IL-13
180
120
60
0
**
IFN-γ
40
30
20
10
0
WT
RhoGAP-/-
WT
RhoAGAP-/-
OVA
PBS

Fig. E3